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Ultrastructure and Physiological Response of Leucophores of the Medaka *Oryzias latipes*

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ABSTRACT—Ultrastructure and physiological responses of leucophores in isolated scales of the medaka *Oryzias latipes* were studied. Many of the leucophores are in close association with overlying melanophores, and nerve fibers that run between the two cells frequently form synapses on both sides. This situation provides a very efficient way to conduct body lightening response, since the stimulation of single adrenergic fiber produces pigment aggregation in the melanophore and dispersion in the leucophore almost simultaneously. Although it appears to be rather infrequent, some nerve fibers enter into the cell body of leucophores. Spherical and tubular synaptic vesicles, and larger vesicles with an electron-dense central core are observed in single nerve fibers. Responses of leucophores are produced by selective migration of the pigment granules toward or away from the center of the cell. Numerous microtubules and 10 nm filaments run parallel to the long axis of the dendrites, though direct connection between these cytoskeletal elements and pigment granules has not been ascertained by electronmicroscopy. Pigment dispersion and aggregation proceed normally in the presence of cytochalasin B while colchicine and EHNA (erythro-9-3-(2-hydroxynonyl)adenine) potently inhibit pigment aggregation. NEM (N-ethylmaleimide) interferes with the pigment dispersion elicited by epinephrine. These results suggest that the intracellular movements of pigment granules in leucophores are microtubule-dependent.

INTRODUCTION

Leucophores in the integument of the medaka are generally found in close association with overlying melanophores to form the melanophore-leucophore combination. Pigment cells in this teleost are under the control of adrenergic nerves, and the prevailing alpha adrenergic receptors on melanophores make their pigment aggregate in response to nervous stimulation producing body lightening [1] while leucophores, which are predominantly controlled by beta adrenergic receptors respond to the same signal with pigment dispersal that also enhances body blanching by increasing light reflectance [2, 3]. Thus, the combination of these two types of pigment cells represents an exquisite example of dermal chromatophore unit [4] in fish integument. Studies on the physiological responses of leucophores have so far been carried out at light microscopic level, but the ultrastructural basis of their responsiveness has not yet been clarified.

The present study deals with the ultrastructure of leucophore and melanophore-leucophore combination. Innervation of nerve fibers into leucophores and melanophore-leucophore combination is clearly shown for the first time. Leucophores contain numerous spherical pigmentary organelles that selectively migrate either centripetally or centrifugally upon aggregative and dispersive stimuli. Their cytoplasm possesses a moderately developed microtubule system in addition to 10-nm filaments. Pharmacological study indicates that the microtubule system, rather than actin-myosin system, is involved in the motile mechanism.

MATERIALS AND METHODS

Leucophores in the dermis of adult *Oryzias latipes* were used in this study. Scales were plucked from the anterior-dorsal region.

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Electron microscopy

Scales were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 30 to 60 min at room temperature, post-fixed in 1% OsO_4 in the same buffer for 30 min at room temperature, dehydrated through a graded series of alcohol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a JEOL 100S electron microscope at the accerelation voltage of 80 kV.

Physiological responses and chemicals

Physiological and pharmacological studies were made on the materials from which overlying epidermis had been removed by a 30 min treatment in 0.25% collagenase (Worthington, type II) in a Ca-free teleost saline solution with gentle agitation. Response of leucophores was observed under a Nikon inverted microscope (Diaphot). Teleost saline solution contained 128 mM NaCl, 2.6 mM KCl and 1.8 mM $CaCl_2$and buffered with 5 mM Tris-HCl at pH 7.2 [3]. Epinephrine (Sigma), theophylline (Tokyo Kasei), colchicine (Sigma), N-ethylmaleimide (NEM, Kokusan Kagaku, Tokyo), 2, 4-dinitrophenol (Tokyo Kasei), potassium cyanide (Kokusan Kagaku), erythro-9-′3-(2-hydroxynonyl)adenine (EHNA, Burroughs Wellcome) and synthetic teleost melanophore concentrating hormone (MCH), which was generously supplied by Dr. M. E. Hadley of University of Arizona, were dissolved directly in the saline solution at appropriate concentrations. Stock solution of cytochalasin B (Aldrich) was made in dimethylsulfoxide (DMSO) and diluted with saline immediately before use.

RESULTS

Morphology of leucophore and its association with melanophore

Although a few leucophores in the dorsal skin are found without having any obvious contact with other pigment cells, a large number of leucophores are observed immediately below overlying melanophores. The dendritic processes of melanophores occasionally extend downward and embrace the upper portion of leucophores. Figure 1 depicts an example where the structures like

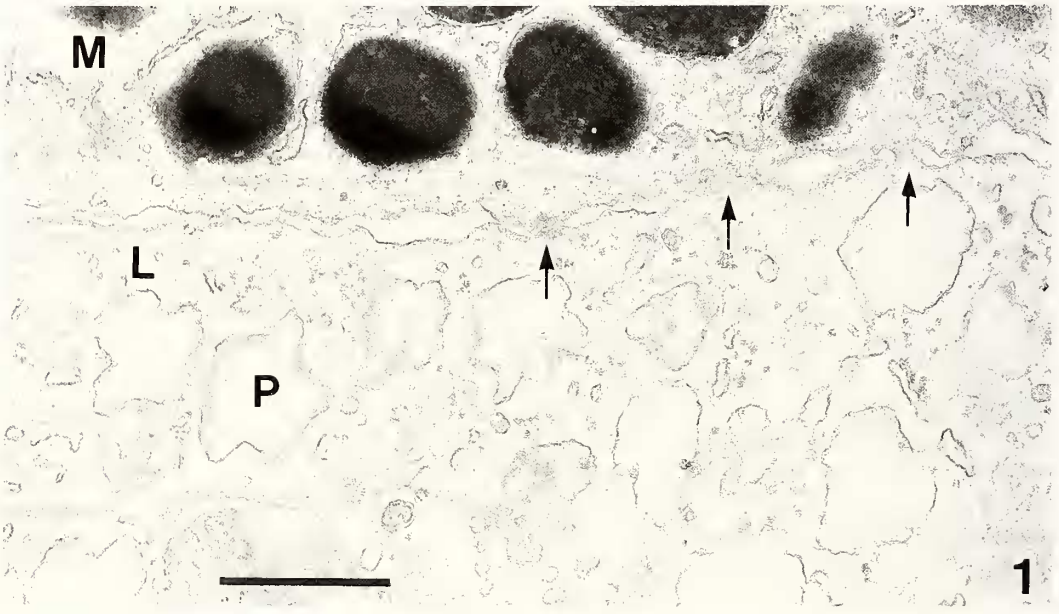


FIG. 1. Overlying melanophore (M) is in close contact with a leucophore (L) to form a melanophore-leucophore complex. Electron lucent vesicles in the leucophore (P) are pigmentary organelles characteristic of this cell type. Arrows indicate the sites where the pigment cells are closely attached. Bar represents 0.5 μm.

intercellular bridge and the accumulation of dense materials on the membranes of both chromatophores are seen (arrows). Though the cells are very closely attached, there is no evidence that indicates the actual cytoplasmic connection between the two different types of chromatophores.

Innervation to melanophore-leucophore combination

Axons with putative synaptic vesicles of various morphology are frequent near the chromatophore combination. Many of them run in the intercellular space between melanophore and leucophore (Figs. 2 and 3) while some enter deeper into leucophores (Figs. 4 and 5). In any case, morphological specialization of pre- and postsynaptic membranes is not distinct. Synaptic vesicles are either spherical or tubular and contain moderately electron dense material. Some of the larger vesicles contain electron dense core. The diameter of smaller vesicles and tubules is about 40 nm while the larger ones with central electron dense core mesure about 80 nm. These three types of synaptic vesicles occur in a single axonal fiber. Although melanophores have a relatively large number of synapses both on the outer (epidermal side) and lower (leucophore side) surfaces, axons on leucophores are rather infrequent compared with those locating on melanophores.

Morphology of leucophores with aggregated and dispersed pigment granules

Figure 6 depicts a typical profile of a leucophore with aggregated pigments. Central portion of the cell is occupied by an aggregate of spherical pigmentary organelles that contain some fuzzy, amorphous intravesicular substance. Pigmentary organelles are rather uniform in size (about 500 nm in diameter). The central cytoplasm contains ER, ribosomes, microtubules, 10-nm filaments and some other membrane-bound vesicles but mitochondria are always found in the dendritic processes. The dendrites are more or less flattened but retain their width after the withdrawal of the pigment. Smaller vesicles, mitochondria, a large number of ribosomes, 10 nm filaments and numerous microtubules aligned parallel to the long axis of the processes are prominent cytoplasmic organelles in dendrites. Golgi complexes are frequent in their proximal portion. In cells with dispersed pigment, dendrites contain pigmentary organelles in addition to the larger vesicles in various size and morphology (Fig. 7). The origin and function of these larger vesicles are unknown, but they sometimes contain intravesicular material similar to that found in pigment granules. A few mitochondria are seen in the central portion of the cell, though the majority of them are densely populated in the proximal portion of the dendrites. Microtubules and 10-nm filaments are also abundant in this region. These findings indicate that chromatophore responses of the leucophores are produced by the change in the intracellular distribution of the pigmentary organelles.

The effects of chemicals on leucophore responses

EHNA Leucophores with their pigment aggregated in the saline solution responded to EHNA with pigment dispersion in a dose-dependent manner. This response was completely reversed by washing the specimens with physiological saline solution. At concentration of 30 μM, EHNA had no appreciable effect on aggregated leucophores, at 60 μM, however, it produced a slight pigment dispersal within 10 min. A 10 min incubation in 125 μM EHNA produced an almost full dispersion of leucophores in 10 min. In 500 μM or 1 mM, dispersal was induced within 5 min. Figure 8 shows an example where aggregated leucophores in saline solution (Fig. 8a) became dispersed after 20 min incubation in 1 mM EHNA (Fig. 8b). Subsequent perfusion with 10 μM epinephrine for 15 min in the presence of EHNA did not change their morphology in appreciable degrees (Fig. 8c). Further treatment of the cells with an aggregating agent, melatonin (1 μg/ml) for 25 min produced only a very minute response (Fig. 8d). The effect of EHNA was reversed by washing the scale in saline solution for 20 min and leucophores became punctate (Fig. 8e). These cells responded to epinephrine in 10 min as shown in Figure 8f.

Colchicine Most of the leucophores in isolated scales remained aggregated in physiological saline. Transfer of these scales into 1 to 5 μM

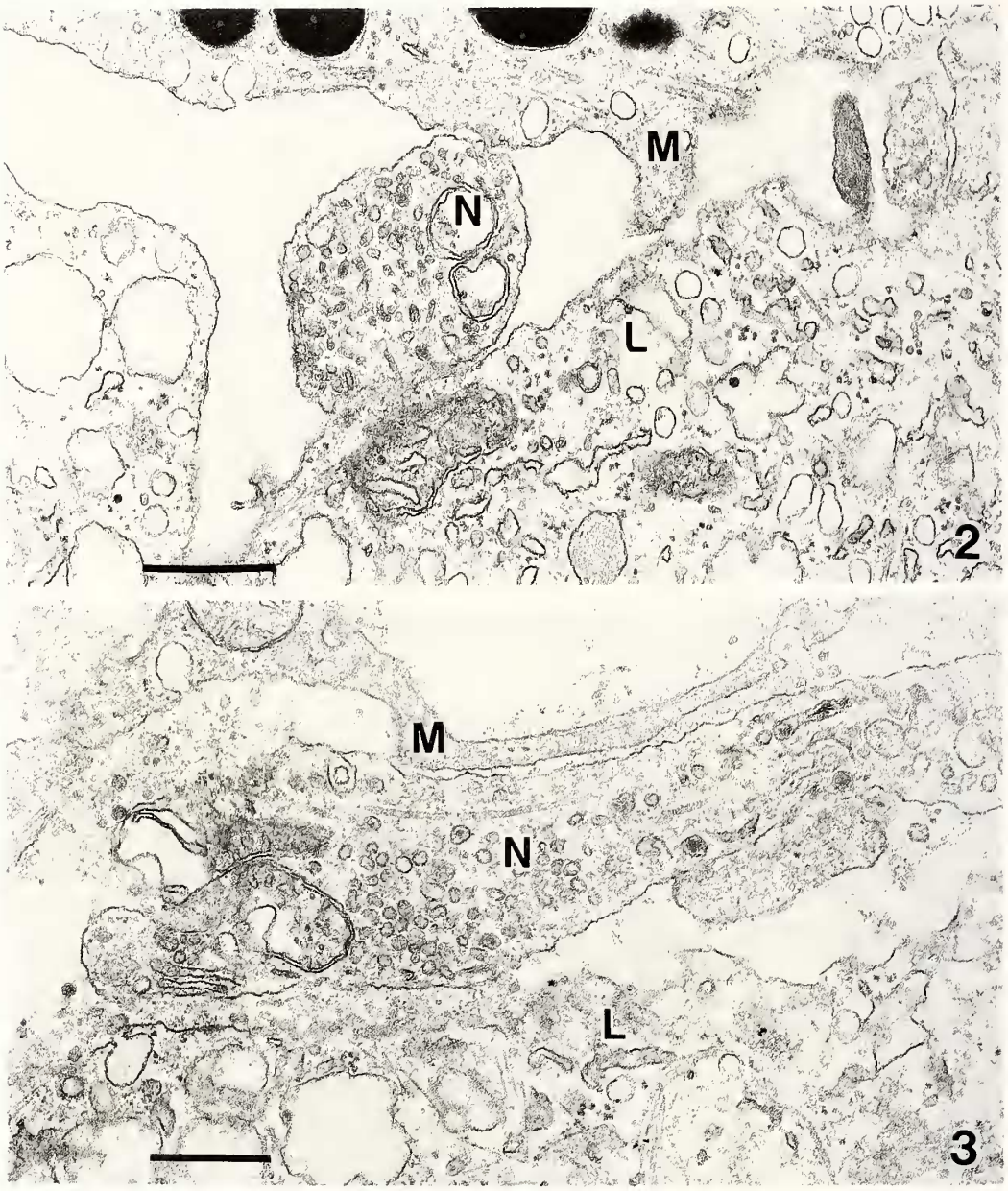
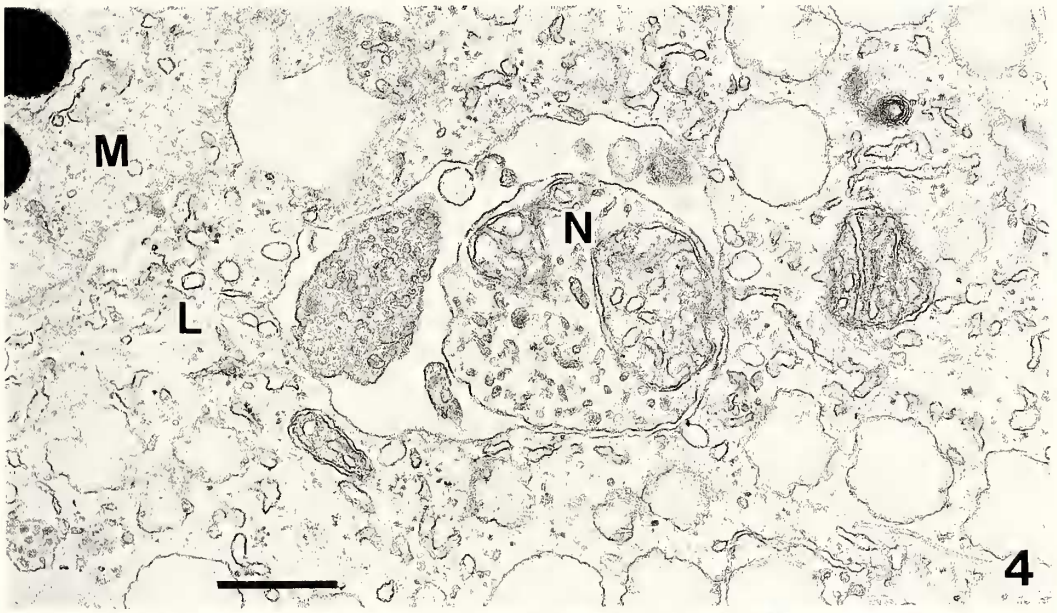


FIG. 2. A single nerve fiber (N) makes synaptic contacts with melanophore (M) and leucophore (L). Bar represents 0.5 μm.

FIG. 3. A longitudinal section of a nerve fiber (N) between the dendrites of melanophore (M) and leucophore (L). Melanosomes are withdrawn from the dendrite. Synaptic vesicles of various size and morphology are seen. Ten nm filaments are found in leucophore. Scale bar represents 0.5 μm.

colchicine produced a rapid dispersion of leucophores within a few minutes. Figure 9 shows leucophores in saline (Fig. 9a) and after 1 hr incubation in 1 mM colchicine (Fig. 9b). Melatonin, epinephrine and theophylline all failed to elicit further response of leucophores in the



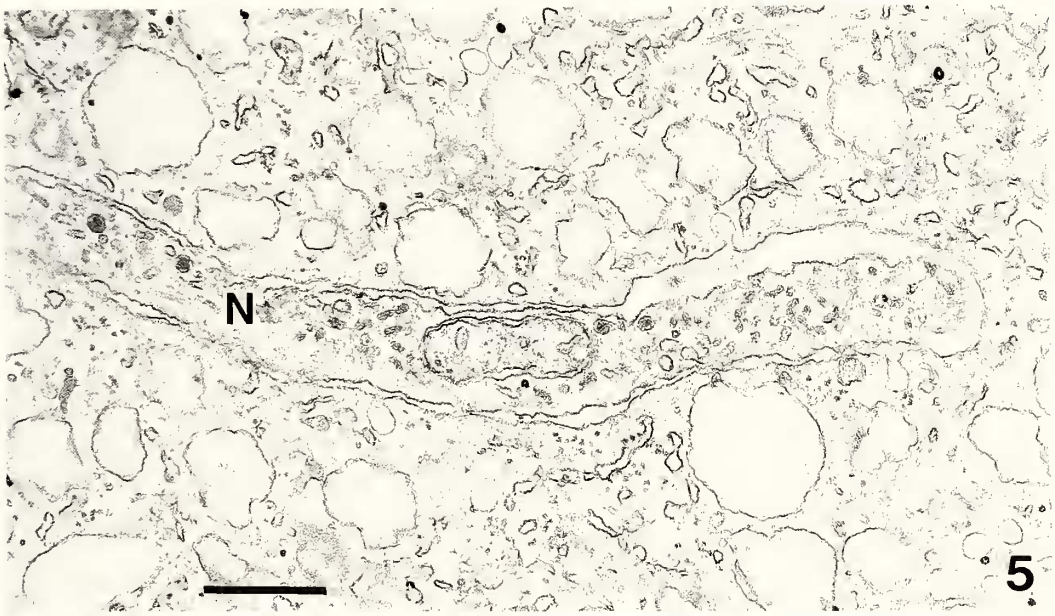


FIG. 4. Cross-sectional profile of a nerve fiber (N). The fiber is encircled by leucophore (L) membrane. Bar: 0.5 μm.

FIG. 5. Longitudinally sectioned nerve (N) found near the central portion of a leucophore. Bar: 0.5 μm.

presence of colchicine.

Cytochalasin B Leucophore responses are found to be totally insensitive to cytochalasin B. Specimens treated in 10 μg/ml cytochalasin B (final concentration of DMSO was 0.25%) for up to 3 hr responded normally to epinephrine with pigment dispersion. Removal of epinephrine produced leucophore reaggregation. Figure 10 shows a typical response of leucophores to cytochalasin B. Most of the leucophores in an isolated scale

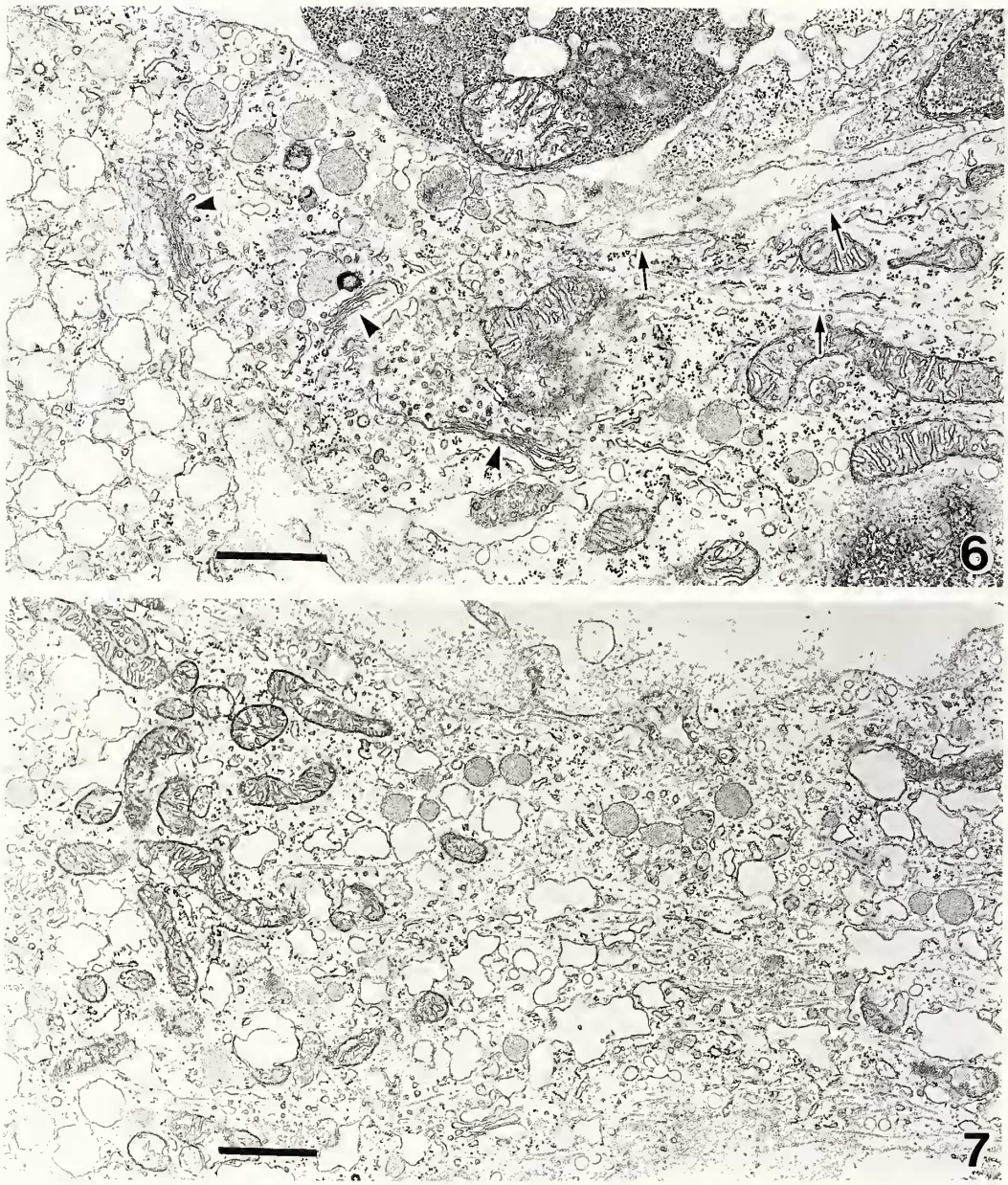


FIG. 6. Proximal portion of a dendrite of a leucophore with aggregated pigment. Pigment granules have migrated toward the cell center (far left), and the dendrite contains Golgi apparatus (arrowheads), mitochondria, microtubules (arrows) and other cytoplasmic organelles except pigment granules. Scale bar represents 1 μm.

FIG. 7. Proximal portion of a dendrite of a leucophore with dispersed pigment. Pigment granules and some larger vesicles are now present in the dendrite. Bar: 1 μm.

remained punctate in saline solution (Fig. 10a). Incubation in cytochalasin B for 2 hr did not change their shape (Fig. 10b) and the following perfusion with the medium containing both epinephrine and cytochalasin B produced a prompt dispersal of the cells (Fig. 10c) within 7

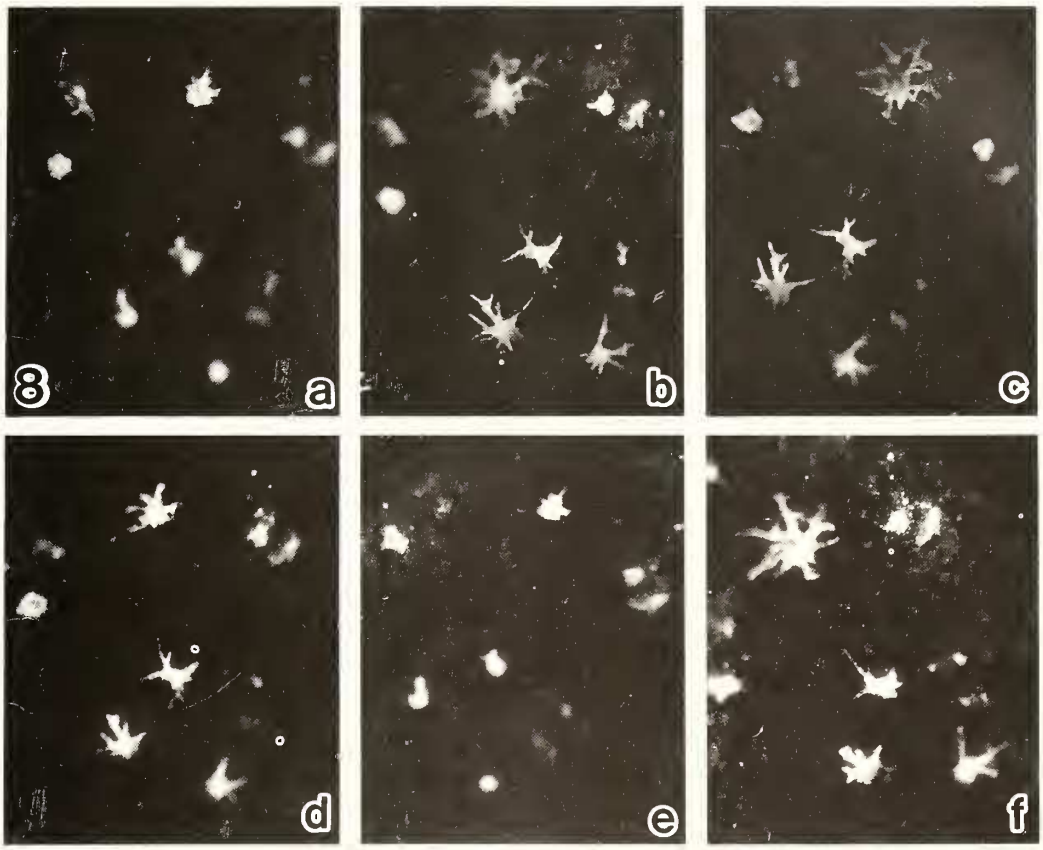


FIG. 8. Effect of EHNA on the physiological response of leucophores. Aggregated leucophores in the saline solution (a) became dispersed by a 20 min incubation in 1 mM EHNA (b). No further dispersion was induced by a 15 min treatment with 10 μM epinephrine (c). These cells did not respond to melatonin in appreciable degrees (d). The dispersive effect of the drug was completely reversed by washing in the saline (e), and the cells rapidly redispersed when treated with epinephrine (f). $\times$113.

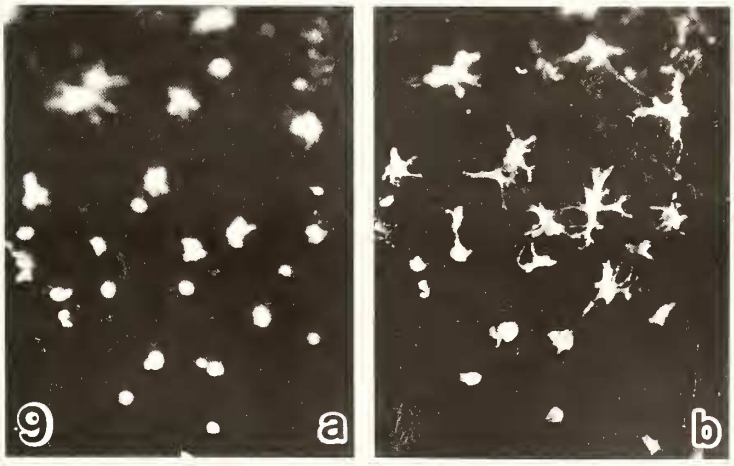


FIG. 9. Effect of colchicine. Aggregated leucophores in the saline solution (a) became dispersed by a 1 hr incubation in 1 mM colchicine (b). $\times$113.

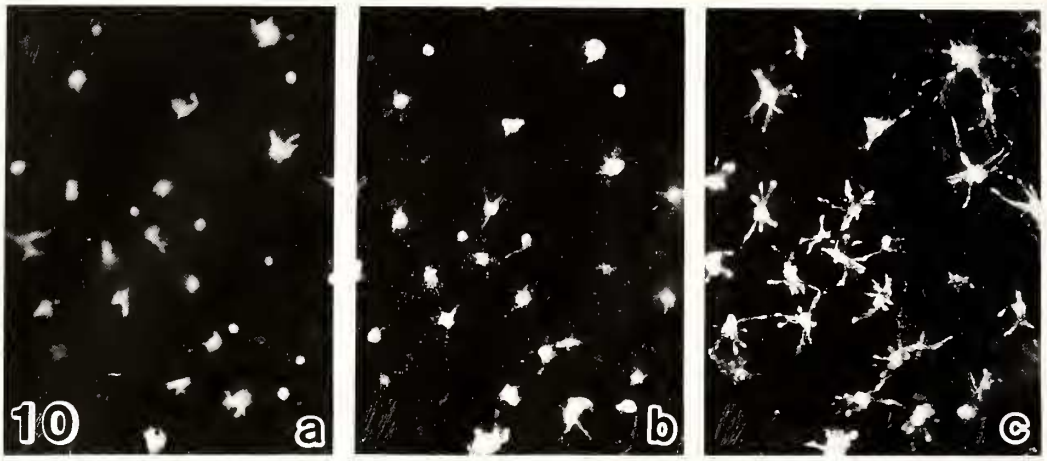


FIG. 10. Effect of cytochalasin B. Aggregated leucophores in the saline (a) remain aggregated during a 2 hr incubation in cytochalasin B (b). Rapid pigment dispersion was induced by epinephrine in the presence of cytochalasin B (c). ×113.

min. Close observation of the dendritic processes of dispersed leucophores indicated that their tips were more inflated compared to those dispersed in the absence of cytochalasin B.

NEM NEM at concentrations between 0.1 to 2.5 mM potently inhibited epinephrine- or theophylline-induced pigment dispersion in leucophores. Application of the drug on dispersed leucophores (produced by theophylline-pretreatment) did not induce pigment aggregation either. This drug has also a very potent inhibitory effect on melanophore aggregation that is normally produced by epinephrine and MCH.

Potassium cyanide and dinitrophenol (DNP) When the isolated scales were incubated in KCN at 5×10^{-4} M for 40 to 60 min, dispersion of leucophores by epinephrine or theophylline was only partially inhibited. DNP at 1 mM potently inhibited leucophore dispersion by epinephrine and theophylline, but produced pigment aggregation when applied on dispersed leucophores. The effects of these metabolic inhibitors on leucophore responses were, however, not as prominent as those found on melanophores and xanthophores where the responses appear to be more susceptible to these chemicals.

Leucophore response to MCH

Leucophores with aggregated pigments produced by placing scales in physiological saline, or those with dispersed state induced by theophylline treatment, were perfused with synthetic MCH (1 nM–1 μM). Neither pigment dispersion nor aggregation was induced although the hormone was potent enough to produce full pigment aggregation in neighboring melanophores within a few minutes at the lowest concentration employed.

DISCUSSION

Membrane specialization of melanophore-leucophore junction

Although leucophores and melanophores of *Fundulus* are in close association as in the present species, no specialized junctional structure has been demonstrated in the earlier work [5]. In *Oryzias,* leucophores and melanophores appear to be more closely associated. Sometimes membranes of the two cells appeared to be tightly attached. Whether the association is simply holding the two cells together (tight junction) or it actually functions as electrical or metabolic coupling (gap junction) remains to be investigated.

Innervation into melanophore-leucophore combination

Pharmacological evidence indicates that leucophores of the medaka are under the control of beta adrenergic receptors [2, 3]. Since rhythmic

pigment granule aggregation and dispersion (pulsation) induced by Ba ions occur simultaneously but in opposite directions in a melanophore-leucophore combination, it has been suggested that the two chromatophores are innervated by the same nerve. Furthermore, the evidence is presented that xanthophores are also controlled by the same nerve, thus providing an efficient way to adapt the fish to its environmental background [6]. Since the early work of Ballowitz [7], innervation into fish melanophores has been studied repeatedly at light microscopic level (see [8, 9] for review). Adrenergic innervation to melanophores and erythrophores has also been demonstrated recently by light microscopic autoradiography [10–12]. At electron microscopic level, chromatophore-neural junctions have been described in the melanophores of *Fundulus* [13], *Chasmichthys* [14] and the angelfish *Pterophyllum* [15]. However, innervation to bright-colored chroamtophores, i.e. xanthophores and leucophores, has not been studied at ultrastructural level. The present study clearly shows that the melanophore-leucophore combination is, in fact, innervated by a single nerve fiber as Iwata and his collaborators have concluded from their physiological studies [6]. The synaptic structure is rather indistinct, membrane specialization with pre- and postsynaptic densities being only occasionally observed. Synapses are frequently found on the epidermal side of melanophore membrane in addition to those found in melanophore-leucophore junctions. Sometimes fibers run deeper into melanophores or leucophores. Observation on serial sections indicates that nerve fibers form en passant synapses as has been shown in the angelfish melanophores [15].

Morphology of leucophore with aggregated and dispersed pigments

Ultrastructural observation revealed that, in contrast to the response of melanophores where the cytoplasm other than pigment granules translocates simultaneously [16, 17], aggregation or dispersion of leucophores is produced by a selective translocation of pigmentary organelles in the cytoplasm toward the centripetal or centrifugal direction. Very selective movement of pigment granules conducted by a cytoskeletal meshwork has been reported in erythrophores [18]. In leucophores, however, direct association of the pigment granules with cytoplasmic microtubules or other cytoskeletal elements has not been demonstrated. Dendritic processes of a leucophore with aggregated pigments contain numerous microtubules, 10 nm filaments, free and membrane bound ribosomes, mitochondria and other membrane bound organelles but are entirely free of pigment granules. The central portion of the cell is, on the other hand, largely occupied by pigment granules but contains other cytoplasmic organelles in much less number compared to the peripheral region. Microtubules running parallel to the long axis of the dendrite are abundant while 10 nm filaments are more frequent near the central portion of the cell. Sometimes small bundles of 10 nm filaments were found at the boundary between the mass of pigment granules and the base of the dendrite. Mitochondria of leucophores, unlike those of melanophores that aggregate toward the perinuclear region during pigment aggregation, did not change their distribution pattern drastically during pigment migration. This suggests that the pigment migration in leucophores proceeds more gently and selectively, provided that mitochondria translocate passively in both cases.

Mechanism of pigment migration

EHNA, a dynein-ATPase inhibitor, made leucophore disperse at concentrations as low as 60 μM. The effect was dose-dependent and was readily reversed by washing. Both in intact and detergent-treated, permeabilized preparations of *Fundulus* melanophores, EHNA at 2 mM blocked epinephrine-elicited pigment aggregation [19] and in melanophores of *Oryzias*, it inhibited epinephrine or MCH-induced melanosome aggregation at concentrations between 0.25 to 2 mM [20]. In *Holocentrus* erythrophores, the drug at 1 to 4 mM prevented the saltatory movement of pigment granules but epinephrine did induce pigment aggregation at slower rate than in untreated cells [21]. Thus, the response pattern of the leucophores to EHNA is similar to that observed in melanophores, though leucophores appear to be more sensitive to the inhibitor.

Kinesin, a microtubule-dependent motor molecule that supports anterograde axoplasmic transport of cytoplasmic particles [22, 23], is reported to be relatively resistant to the effect of EHNA [24] or NEM [23]. The movement of pigment granules within leucophores in centripetal and centrifugal directions was totally arrested by the presence of NEM, and centrifugal displacement of the granules was produced by EHNA. Although the effects of these drugs and those of uncouplers of oxidative phosphorylation may be partially due to the depletion of ATP, relatively high sensitivity of the leucophores to NEM and EHNA suggests the involvement of dynein-tubulin interaction in their responses, especially in pigment aggregation, as has been suggested in fish melanophores [19, 20] and melanoma cells [25]. The dispersive effect of colchicine is common to all types of *Oryzias* chromatophores but hardly reversible. Pigment dispersion in leucophores was induced rapidly in the presence of relatively low concentrations of the alkaloid. Cytoplasmic microtubules appear to be resistant to this drug, and a derivative of colchicine, lumicolchicine, which does not bind to tubulin also blocks pigmentary responses in melanophores in a similar manner as colchicine does [26]. These observations suggest that the site of action of colchicine is not restricted on cytoplasmic microtubules. The involvement of actin-myosin system in the pigment movements is not likely since the responses were insensitive to cytochalasin B.

It has recently been suggested that pigment dispersal and aggregation in fish xanthophores and melanophores are brought about by protein phosphorylation and dephosphorylation, respectively [27–30]. Though the mechanism of pigment translocation in leucophores remains to be ascertained from this point of view, induction of pigment dispersal by theophylline or dibutylyl cyclic AMP [2] allows an assumption that the elevation of intracellular cyclic AMP level produces pigment dispersal in leucophores as well as in the other types of chromatophores.

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